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# Foreign Gene Expression in the Mouse Testis by Localized *in Vivo* Gene Transfer

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**In order to attain foreign gene expression *in vivo* in the testis of living mice, chloramphenicol acetyltransferase (CAT), firefly luciferase and bacterial lacZ reporter genes were transfected by microparticle bombardment and electroporation. The results showed that CAT reporter gene was expressed in a dose-dependent fashion. The X-gal staining showed that in some spermatogenic-like cells, the bacterial lacZ gene was also expressed by *in vivo* electroporation, but not by *in vivo* microparticle bombardment. The possibility of *in vivo* gene transfer to the spermatogenic cells of the mouse testis was further confirmed by the fact that the CAT reporter gene expression was testis-specific when driven by the mouse-protamin 1 promoter. It was concluded, therefore, that *in vivo* microparticle bombardment and, especially, electroporation provide convenient and efficient means of gene transfer to the testis of living mice.** © 1997 Academic Press

Until today, gene transfer to germ cells has been attempted by a variety of methods including microinjection, embryonic stem cell mediated transfection, virus mediated transfection, and sperm mediated transformation (1). The last method is of particular interest because of its ease and simplicity. Simple mixing of sperms with foreign DNA followed by artificial insemination was reported to result in successful generation of transgenic mice (2) (for review on this topic, see ref.3). However, a serious doubt has been cast about the sperm mediated gene transfer that the method is not reproducible, and therefore is of little importance in practice (4).

Instead of the above sperm mediated gene transfer, direct introduction of DNA into male germ cells could provide a new alternative strategy for producing transgenic animals. Recently, the idea of localized *in vivo* gene transfer (LIVGET) has become popular because of increasing numbers of gene therapy trials. Foreign genes could be experimentally transferred *in vivo* to living animals by several LIVGET methods such as virus mediation, lipofection, microparticle bombardment (MPB) and implantation (5).

In terms of applicability to a broad range of cells and tissue, simplicity, and relatively low risk of biohazard, much attention has been focused to physical methods such as MPB (6). Several reporter genes have been transferred to somatic tissues of live animals using devices based on gunpowder, electric discharge, and high pressure gas (7-9). The expression of these genes thus introduced has been detected in various tissues such as skin, liver and muscle. Accordingly, it should be possible to apply the same MPB technology to transfer foreign genes to the testis of living animals *in vivo*, and thereby hopefully producing sperms that carries foreign genes.

With respect to physical LIVGET means, electroporation (EP) is also considered to have high potential since it can deliver antitumor drugs and antibodies locally to various types of cancers (10-13). There is no reason why the same EP method cannot be applied to deliver genes to target cells and tissues *in vivo* in place of drugs or antibodies. Indeed, the LIVGET by *in vivo* EP to the rat brain, chicken embryo, and the mouse testis was reported (14-17). The advantages of *in vivo* EP over MPB are: possible tissue damage is less ; there is no limitation of DNA to be transfected at a time, and ; DNA can be transferable to cells deep inside the target tissue, but not so by *in vivo* MPB.

We conducted the present study in an attempt to transfer foreign DNA to the testis of living mice *in vivo*. The results indicated that although gene expression was deemed most probably transient, LIVGET methods by MPB and EP could provide convenient means of gene transfer to spermatogenic cells in the testis of living mice.

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Abbreviations: LIVGET, localized *in vivo* gene transfer; MPB, microparticle bombardment; EP, electroporation.



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## MATERIALS AND METHODS

*Animals.* ICR strain mice at 4 to 5 weeks of age were used in all experiments. They were cared for under Guideline of Animal Experimentation, laid down by the Committee of Experimental Animal Care, Nagoya University, Nagoya, Japan.

*In vivo DNA transfection.* For *in vivo* MPB, skin surrounding the testis was excised under the light anaesthesia, and the testis was exposed. Subsequently, a small area of the testicular capsule about 2 × 2 mm was excised. Usually 5 mg tungsten of a median particle size of 0.36 μm (Bio-Rad, USA) with 0.83 μg DNA was placed onto a cylinder-like aluminium bullet 5 mm tall and 5 mm in diameter, and shot with the Machimpacter (Kansai Paint, Osaka, Japan) at the capsule-removed target area by the pressure of nitrogen gas at 80 kgf/cm² at a distance of 3 cm from the nozzle. Where necessary, the amounts of DNA were varied from 0.42 to 10 μg per bullet. After the *in vivo* MPB, the skin was stitched, and the mice were then maintained for the subsequent 48 hrs.

For *in vivo* EP, similar surgical procedures were carried out to expose testicular tissues except for the removal of the capsule. Designated doses of plasmid DNA, from 5 to 150 μg per testis, dissolved in TE buffer (10 mM Tris, and 1 mM EDTA, pH adjusted to 7.5), were injected into the left testis with a 1 ml syringe and a 27G needle. Immediately after the injection, square electric pulses were applied eight times with an electro-square porator T820 in combination with an optimizor 500 (BTX, San Diego, USA) at 25 V with a time constant of 50 msec. Where necessary, voltages and time constants were varied. After the *in vivo* EP, the skin was stitched, and the mice were maintained for the following 48 hrs.

The plasmid DNAs used were pCAT control (Promega, USA) for expressing CAT activities, pmp1CAT for evaluating testis-specific expression (18,19), pmiwZ for histochemical staining of testicular cells (20), and pmiwluc for normalization of transfection efficiency.

For testis-specific expression of the CAT reporter gene, pmp1CAT was constructed from the product of PCR amplification of the DNA fragment encoding the mouse protamin 1 gene promoter from −1.7 kb to +1 bp relative to the cap site based on the plasmid, pmp1-3.2 which was kindly gifted from Dr. R. D. Palmiter, Howard Hughes Medical Institute, Department of Biochemistry, University of Washington, USA. The primers used for the PCR reaction were as follows: sense primer, 5'-AGTGCC**AAGCTT**GCATGCCTGCA-3' (23 mer); antisense primer, 5'-GACTCT**AAGCTT**GAGCAGGTGGAATTT-3' (27 mer), where underlined and bold letter sequences indicate the restriction site of Hind III and the complimentary site of the pmp1-3.2, respectively. The amplified 1.7 kb fragment was inserted into the Hind III and Xho I sites of the pOVCAT-.1 vector (21) from which the corresponding DNA fragment located between these restriction sites had been removed prior to ligation. The plasmid, pmiwluc, used for normalization of transfection efficiency, was constructed by inserting the BamHI fragment of pSVluc into the same restriction sites of pmiwZ (20).

*Assays.* For analyzing the *in vivo* transfected samples, the mice were sacrificed by decapitation at 48 hrs after the transfection, and the testis samples were excised quickly, and weighed. Whole testis samples were then homogenized with three volumes of buffer A containing 15 mM Tris, 60 mM KCl, 15 mM NaCl, 12 mM EDTA, 1 mM dithiothreitol, 0.15 mM spermine and 0.4 mM phenylmethylsulfonyl fluoride (pH adjusted to 8.0) for measurement of CAT and luciferase activities. For the measurement of CAT but not luciferase activities, heating treatment at 70 °C was conducted for 60 min in order to increase the sensitivity as suggested previously (22). The homogenate was centrifuged at 6000 × g for 11 min, and the supernatant was used for measurements of protein content and CAT activity as described previously (23).

For detection of β-gal activity, histochemical staining was used. The testes were fixed with 4% formaldehyde and 0.2% glutaraldehyde solution in phosphate buffered saline (PBS) at room temperature overnight, and rinsed with PBS twice. Soaking in the X-gal reaction mixture (pH adjusted to 7.6, containing 0.1% X-gal, i.e. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5% N,N-dimethylformamide and 0.1% Triton-X dissolved in XG buffer which was composed of 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6.3H_2O$ and $K_3Fe(CN)_6$ in PBS) was conducted for not more than 1 hr to minimize endogenous greenish-blue color development in the entire testis samples. For light microscopic examination, the testis was embedded in paraffin for sectioning and counter-staining with hematoxylin.

To demonstrate testis-specific gene expression, CAT activities between tissue samples were normalized for transfection efficiency based on the expression of the firefly luciferase originating from the co-transfected pmiwluc driven by the miw promoter, which was deemed to confer universal gene expression among various mammalian tissues (20). Luciferase activity was measured with a luminometer (AutoLumat LB953, EG&Berthold, Germany) by mixing 20 μl tissue extract with 100 μl luciferin solution.

*Statistical analysis.* The data were treated statistically by analysis of variance, and significance of differences between means was tested by Duncan's multiple range test by using General Linear Model Procedures (24). Where necessary the data were transformed to their natural logarithmic values to stabilize error variance.

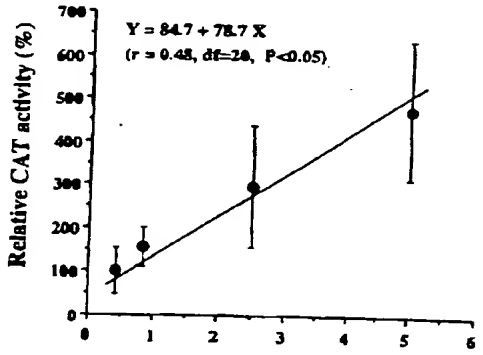


**FIG. 1.** The relationship between CAT activity and dose of DNA used for *in vivo* microparticle bombardment. The regression equation calculated was : Y = 84.7 + 78.7 X (r=0.48, df=20, P<0.05) where Y and X stand for relative CAT activity (expressed as the percentage of the CAT value of the lowest DNA dose at 0.42 μg, 830 dpm/100 μg protein) and the dose of DNA (μg), respectively.

## RESULTS

### *Gene Transfer by in Vivo MPB*

At first, we examined the relationship between the dose of DNA transfected by the *in vivo* MPB and relative CAT activity. The results are given in Fig. 1. The regression equation calculated was : Y = 84.7 + 78.7 X (r=0.48, df=20, P<0.05) where Y and X stand for relative CAT activity (expressed as the percentage of the CAT value of the lowest DNA dose at 0.42 μg, 830 dpm/100 μg protein) and the dose of DNA (μg), respectively. Thus, as the dose of transfected DNA increased, the CAT gene expression elevated linearly.

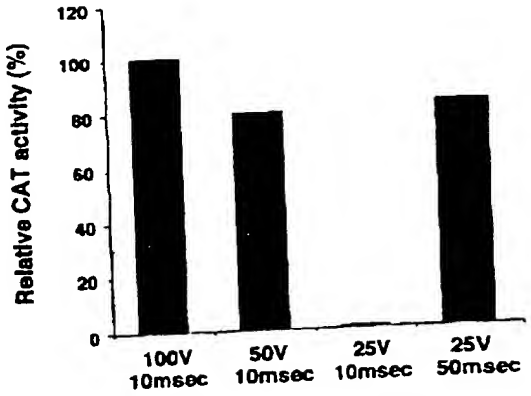


FIG. 2. Effects of varying voltage and time constant on CAT activity expressed as percentages of the mean value for the 100V/10 msec group (8,100 dpm/100 μg protein). The plasmid DNA (pCAT control) was transfected to the mouse testis by *in vivo* electroporation at 10 μg, and CAT activity was determined at 48 hrs after transfection. Electric pulses were applied 8 times at indicated voltages and time constants.

*Gene Transfer by in Vivo EP*

The optimal voltage and time-constant for *in vivo* EP were investigated by varying the two parameters. When severe conditions such as 100 V/50 msec were used, tissue damage was observed due to the heat generated while applying electric pulses. Therefore, in Fig. 2, only the results from those conditions where little tissue damage was found in the testis were presented, and the CAT activities were expressed as the percentage of the mean value for the 100V/10 msec group (8,100 dpm/100 μg protein). Decreasing the voltage from 100 to 50, and especially from 50 to 25 resulted in a sharp reduction in the CAT gene expression, while the extended time-constant from 10 to 50 msec at the same 25 V restored almost completely the CAT activity. Consequently, the combination of either 50 V/10 msec or 25V/50 msec was considered to be optimal for the CAT gene transfection by *in vivo* EP under the present experimental conditions.

By using the *in vivo* EP method, plasmid DNA, pmiwZ that contained the bacterial lacZ gene was transfected at 150 μg, and the gene expression was detected by the X-gal staining of the mouse testis. As shown evidently, a portion of seminiferous tubules was stained with blue color on one side of the testis (Fig. 3A), and less extensively, the other side of the same testis (Fig. 3B), indicating the expression of the bacterial lacZ gene. Light microscopic appearance of cross sections counter-stained with hematoxylin demonstrated that some spermatogonium- and spermatocyte-like cells located close to the basement membrane (Fig. 3C) or spermatid-like elongated cells with flagellum in the middle of the seminiferous tubule (Fig. 3D) were stained blue with X-gal. The same reporter gene, pmiwZ, was also transfected by in vivo MPB at 10 μg/testis which was the maximum dose transfectable at a time by this method, no cells stained with X-gal were detected (data not shown).

*Testis-Specific Expression of the CAT Gene Transferred by in Vivo EP*

In order to further confirm that foreign genes are actually transferred to spermatogenic cells by the LIVGET methods, the CAT gene expression was driven by the spermatid-specific promoter, the mouse protamin (18,19). For this purpose, the CAT activities (ranging from 1,000 to 3,000 dpm/100 μg protein) were normalized for transfection efficiency by the expression of the cotransfected and universally expressed firefly luciferase gene (ranging from 6,000 to 2,000,000 relative right units/100 μg protein), and presented as the percentage of the testis mean value in Fig. 4. As shown clearly, when transfected with pmp1CAT carrying the mouse protamin 1 promoter by *in vivo* EP, CAT gene expression in the testis was predominantly higher than those in other two tissues. The levels of CAT gene expression detected in the leg muscle and liver were low and statistically indistinguishable from the unexpressed mock control level.

## DISCUSSION

Although DNA transfer to male germ cells and testicular cells *in vitro* has already been reported (25,26), the *in vitro* transfection methods appeared to be inefficient (27). Moreover, the calcium phosphate method which has been most commonly used for cultured cells resulted in a drastic reduction in the cell viability ; more than 80% of the cultured mouse spermatogenic cells died within 5 days (27). In contrast to *in vitro* methods the LIVGET techniques have great advantages : they are simple and convenient ; adequate development and differentiation of transfected cells can be maintained in the *in vivo* environment after the transfection, and in principle ; they can be applied to any types of cells and tissues so long as the target is accessible. However in the case of MPB, because of high nitrogen gas pressure used to accelerate the DNA coated tungsten microparticles, serious tissue damage may have occurred. In comparison with MPB, *in vivo* EP appeared to be more suitable for LIVGET technique to the mouse testis because : the possible tissue damage was less ; there was no limitation of DNA to be transfected at a time so long as a desired amount is soluble in a small volume of the buffer whereas the amount of plasmid DNA maximally loaded onto an aluminium bullet in the present MPB apparatus was only 10 μg, and ; DNA could be transferable to cells deep inside the target tissue, which was not possible by *in vivo* MPB. By changing reporter genes from CAT to lacZ, the *in vivo* MPB method fail

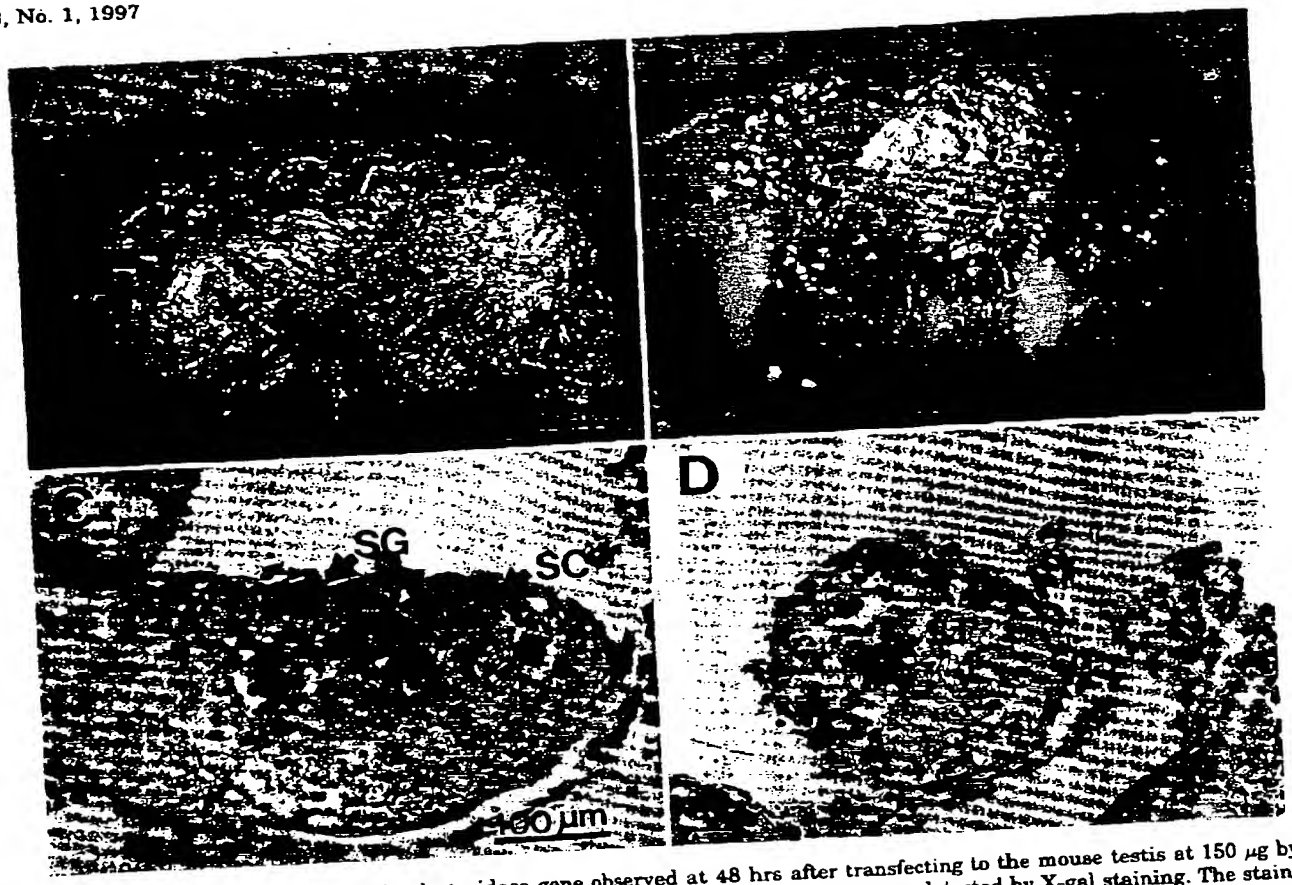


FIG. 3. Expression of the bacterial β-galactosidase gene observed at 48 hrs after transfecting to the mouse testis at 150 μg by *in vivo* electroporation (A,B, whole testis, × 8 ; C,D, cross sections, × 180). The gene expression was detected by X-gal staining. The staining time period was limited to 1 hr to minimize endogenous color development. A portion of seminiferous tubules was stained on one side of the testis (Figure 3A), and the other side of the same testis (Figure 3B), indicating the strong expression of the bacterial lacZ gene. Light microscopic appearance of cross sections counter-stained with hematoxylin demonstrated that some spermatogonium- and spermatocyte-like cells as indicated by arrows located close to the basement membrane (Figure 3C) or spermatid-like elongated cells with flagellum as indicated by an arrow in the middle of the seminiferous tubule (Figure 3D) were stained with X-gal. Abbreviations used: SG, spermatogonium-like cells; SC, spermatocyte-like cells; ST, spermatid-like cells.

to confer distinguishable gene expression (data not shown) partly because of enhanced endogenous greenish-blue color development due to tissue damage, low sensitivity of X-gal staining, and of poor DNA penetration only 2 to 3 mm deep from the surface of the tissue (8). In this regard, the usefulness of *in vivo* EP was substantiated when it was employed. In the X-gal stained testis cross-sectioned, and examined under the light microscope, the bacterial lacZ gene was clearly expressed in some spermatogenic-like cells in seminiferous tubules deep inside the testis.

Unless tissue-specific promoters are used, the reporter gene is likely to be universally expressed in a mixed cell population of a variety of tissues *in vivo*. Thus, if specific gene expression in spermatogenic cells is desired, suitable promoters such as protamin (18,19,28) and hsp70 (29) should be used. Indeed as shown in Fig. 4, we demonstrated the testis-specific CAT gene expression by the use of the mouse protamin-1 promoter as suggested from the study with transgenic mice (19). Again, this indicated the usefulness of *in vivo* EP as the LIVGET method, although whether or not the CAT gene expression driven by the protamin-1 promoter was strictly confinded to spermatids was not examined.

Apart from the tissue- or cell type-specificity, gene expression observed in the present study was deemed transient. Because the integration of transfected DNA into chromosomes occurs at a very rare rate, selection of such DNA-integrated cells by drug resistance *in vivo* or alternatively the transfection of self-replicating plasmid DNA within testicular cells of living mice would have to be employed to attain long-lasting and increased gene expression.

A great deal of technological progress should still be made with respect to the integration of transfected genes, means for selecting spermatids with foreign genes for fertilization, and demonstration of fertiliza-

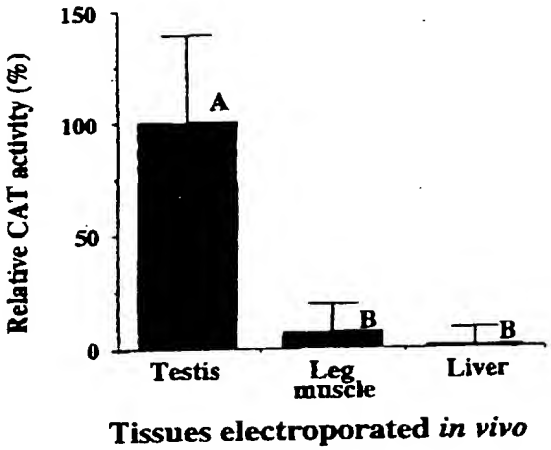


**FIG. 4.** Testis-specific CAT activities after transfecting the CAT reporter gene driven by the mouse protamin 1 promoter (pmp1CAT). The pmp1CAT was transfected to the mouse testis, leg muscle or liver by *in vivo* electroporation, and the CAT activity was determined at 48 hrs after transfection. The CAT activities (ranging from 1,000 to 3,000 dpm/100 μg protein) were normalized for transfection efficiency by the expression of the cotransfected and universally expressed firefly luciferase gene (ranging from 6,000 to 2,000,000 relative right units/100 μg protein), and presented as the percentage of the testis mean value. $^{A,B}$Significantly different at $P<0.01$. Vertical bars represent means ± SEM of 4 replicates.

tion and embryonic integration. Nevertheless, the present results suggest that LIVGET methods by MPB, and especially, by EP could provide means of *in vivo* gene transfer to spermatogenic cells in the testis of living mice. Thus, the LIVGET technology may encourage to bring the spermatogenic cell-mediated gene transformation to completion, a novel approach for the production of transgenic animals.

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